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RESEARCH ARTICLE

Upregulation of circulating cancer stem cell marker, DCLK1 but not Lgr5, in chemoradiotherapy-treated colorectal cancer patients

Alireza Mirzaei · Gholamreza Tavoosidana · Mohammad Hossein Modarressi · Afshin Abdi Rad · Mohammad Sadegh Fazeli · Reza Shirkoohi · Masoumeh Tavakoli-Yaraki · Zahra Madjd

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Abstract Cancer stem cell (CSC) markers have attracted considerable attention in tumor diagnostic, prognostic, and therapeutic implications. Detection of cancer stem cells in circulating blood using cancer stem cell markers has received remarkable attention recently. In this study, we aimed to investigate the messenger RNA (mRNA) expression level of Lgr5 and DCLK1 as most proposed colorectal CSC markers in blood circulation also determine the subsequent association to patients' clinical and pathological findings. Peripheral blood mononuclear cells (PBMCs) of 58 patients with colorectal cancer at stage I–IV with 33 out of 58 patients undergoing preoperative chemoradiotherapy (CRT), as well as 58 healthy controls have been isolated and the extracted RNAs were analyzed using real-time PCR. The mRNA expression pattern of CSC markers of patients and controls was compared using ΔΔCt method. The expression level of Lgr5 was significantly higher in colorectal cancer (CRC) patients comparing to healthy group (4.8-fold change, $p<0.001$). Also there was a significant increase in expression level of Lgr5 in patients at stages III and IV comparing to stages I and II ($p=0.031$) and higher grades ($p=0.039$) of CRC. The expression of DCLK1 was also elevated in patients significantly (2.7-fold change, $p<0.001$) and the related expression was increased by increasing disease stage (p=0.025). Combination of DCLK1 and Lgr5 markers was analyzed by logistic regression and proved to be a slightly better marker compared to each marker alone. Interestingly the DCLK1 expression level was significantly higher in patients undergoing preoperative CRT ($p=0.041$); however, no association to neoadjuvant CRT was observed for Lgr5. Considering the over-expression of DCLK1 and Lgr5 in circulating blood of CRC patients comparing to controls, our results might emphasize on the presence of CSCs in blood of these patients which might be attributed to their

A. Mirzaei · G. Tavoosidana (✉)
Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Eastern side of Tehran University, 88, Italia St, Tehran, Iran
e-mail: g-tavoosi@tums.ac.ir

M. H. Modarressi
Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

A. A. Rad
Surgical Pathology Department, Cancer Institute, Tehran University of Medical Sciences, Tehran, Iran

M. S. Fazeli
Department of Surgery, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran

R. Shirkoohi
Group of Genetics, Cancer Research Center, Cancer Institute, Tehran University of Medical Sciences, Tehran, Iran

M. Tavakoli-Yaraki
Department of Biochemistry, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran

Z. Madjd
Department of Molecular Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Hemmat Street (Highway), Next to Milad Tower, Tehran, Iran

Z. Madjd (✉)
Oncopathology Research Center, Iran University of Medical Sciences, Hemmat Street (Highway), Next to Milad Tower, Tehran, Iran
e-mail: Zahra.madjd@yahoo.com

clinical and pathological characteristics and may lead to apply in future clinical implications. Moreover, the higher expression level of DCLK1 in patients undergoing CRT can propose it as a more relevant candidate among CSC markers comparing to Lgr5 for CRC patients.

Keywords Colorectal cancer · Cancer stem cell · Chemoradiotherapy · DCLK1 · Lgr5

Introduction

Colorectal cancer (CRC) is the third cause of cancer-related death in the world [1], and patients who undergo curative resection are at a high risk of developing local or metastatic recurrence later in their life [2]. In spite of several available diagnostic procedures for early detection and monitoring of CRC including fecal occult blood test (FOBT) and carcinoembryonic antigen (CEA), finding more reliable, sensitive, and specific markers is still considered as a primary concern for prognostic and diagnostic purposes [3]. Recently, a great deal of investigations has focused on cancer stem cell (CSC) and its markers in tumor diagnosis, monitoring, and therapy [4–6]. According to CSC hypothesis, a minority population of cancer cells defined as CSCs is responsible for tumor initiation, metastasis, recurrence, and drug resistance [7–11], suggesting that application of CSC markers is a more efficient strategy for diagnosis, monitoring, and treatment of tumors.

CSCs, like normal stem cells, are characterized by their capability of asymmetric division to make more stem cells and symmetric division to generate rapidly differentiating cells. In other description, CSC population consists of a mixture of two phenotypes: a proliferative and a quiescent type resulting from symmetric and asymmetric cell divisions, respectively [12]. Many recent studies suggest that quiescent CSCs are more invasive CSCs which play the main role in tumor invasiveness, while proliferating CSCs are the main players of tumor bulk formation through uncontrolled symmetric division leading to more proliferating tumor cell population [13–16]. Given the fact that radiotherapy and chemotherapy affect dividing cells, quiescent CSCs resist chemoradiotherapy (CRT) and cause tumor recurrence, sometimes many years later [17–20]. As a result, accepting cancer stem cell concept demands reevaluation and redirection of cancer therapy and diagnosis in order to concentrate our approach on tumor stem cell as a tumor root instead of tumor differentiating cell as tumor foliage.

CSCs have been isolated from several tumors including breast [21], brain [22], prostate [23], pancreas [24], liver [25], and lung [26]. Moreover, many of potential markers including CD133 [27–29], CD44 [29], CD166 [29], and CD24 [27, 30] have been frequently reported as colorectal CSC markers. Although commonality of normal tissue stem cell markers and cancer stem cell markers has always been a controversial challenge in application of CSC markers for clinical implications [31], Nakanishi et al. focused on this drawback and demonstrated that specific tumor stem cell markers are remained to be unraveled [32]. Their investigation revealed that doublecortin-like kinase 1 (DCLk1), a member of doublecortin family (Dcx) which encodes several microtubule-associated proteins (MAPs) [33], distinguishes between tumor and normal stem cells in the intestine. MAPs bind to microtubules and stabilize them [34], therefore helping cell division. Since the life of tumor depends on continuous proliferation of tumor cells, it can be postulated that MAPs are probably involved in tumor progression [35]. In this regard, targeting a MAP that is expressed specifically in cancer stem cells and not in normal stem cells could be the missing key of tumor diagnosis, monitoring, and therapy. Nakanishi and his colleagues also suggested that DCLK1 and other CSC markers of CRC named leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) are expressed simultaneously, and they further showed that $DCLK1^{+}/Lgr5^{+}$ cells have the potential to be considered as CSC of colorectal cancer. Lgr5 also known as G protein-coupled receptor 49 (GPR49) has been recently notified as a marker of intestinal stem cell [36] and CSC [37–39].

Accumulating evidences suggest that CSC is the main subpopulation involved in invasive properties of tumors such as metastasis [40]. Based on the classical metastasis simplification, an orderly sequence of steps, including local invasion, intravasation, survival in the blood circulation, extravasation, and colonization in new organ, is expected in order to complete the metastatic cascade [41, 42]. Assuming CSCs as the most qualified cells to form metastasis, tracing them in the blood circulation step would be a valuable consideration.

Based on these findings, we hypothesized that DCLK1and Lgr5 expression in peripheral blood (PB) might reflect the presence of circulating stem cells in CRC. Thus, we aimed to trace these markers in blood sample of CRC patients to elucidate the probable potency of these two genes as potential CSC markers.

Since DCLK1 and Lgr5 are frequently cited to be the most potential CSC markers in CRC [43–46], a reasonable correlation between the expression of DCLK1 and Lgr5 in blood circulation is expected.

In consequence, our study focused on the evaluation of DCLK1 and Lgr5 expression in the PB of colorectal cancer patients using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). First, we aimed to find out whether these markers are detectable in blood samples of CRC patients which may further apply in diagnostic or prognostic settings. Second, we explored the existence of reasonable correlation between the expression patterns of these two markers and clarified whether they are attributed to patient's clinicopathological characteristics. Possible associations between these two markers were assessed in order to find whether their combination may lead to the more efficient marker.

To our knowledge, this is the first study that is exploring the expression level of DCLK1 as a potential CSC marker in the PB of CRC patients and revealing its association to Lgr5 as another proposed CSC marker of colorectal cancer.

Materials and methods

Patients

A total number of 58 CRC patients, 34 with colon and 24 with rectum cancer, from surgery department of Imam Khomeini Hospital of Tehran University of Medical Sciences were included in this study. The patients' clinical characteristics are shown in details in Table 1.

Tumors were staged and graded according to TNM classification [47], and 11 patients were at stages I and II, 28 cases were at stage III, and 19 patients were at stage IV of adenocarcinoma.

The same number of control samples was taken from patients or persons who were referred to hospital for other reasons with the exception of any kind of cancer. Controls with the history of any kind of tumor were excluded from the study. Controls' age and sex distribution was considered to be comparable to the patients. The mean age of patients and controls was 64.6 and 63.8, respectively.

Table 1 Patients' clinicopathologic characteristics

Parameter	Groups	Number	Percent
Age (years)	20 to 40	9	15.5
	40 to 60	23	39.7
	≥60	26	44.8
Gender	Female	27	46.5
	Male	31	53.5
Tumor location	Colon	34	58.6
	Rectum	24	41.4
TNM stage	I and II	11	19
	III	28	48.3
	IV	19	32.7
Grade	Low	16	27.5
	High	43	72.5
Number of metastatic sites	0	39	67.2
	1	14	24.2
	≥2	5	8.6
Lymphatic invasion	Unknown	7	12.1
	No	16	27.5
	Yes	35	60.4
Neoadjuvant history	Yes	33	56.9
	No	25	43.1

Considering the fact that preoperative CRT can have positive effect on tumor control and morbidity reduction in locally advanced tumors [48, 49], neoadjuvant CRT were administered for stages II to IV based on the protocol proposed by a German rectal cancer study group [49]. The main neoadjuvant chemotherapy regimen consisted of intravenously infusion of a dose of 1000 mg/m^2/day of 5-fluorouracil (5-FU) during the first and fifth weeks of radiotherapy. The concurrent preoperative radiotherapy protocol included the administration of a total dose of 50 GY which was given in 25 fractions during 5 weeks. Totally, 33 out of 58 patients were preoperatively CRT treated.

This study was approved by the Ethics Committee of clinical investigation of Tehran university of Medical Sciences, and written informed consents were obtained from all patients and controls.

Samples A total of 10 ml blood sample was withdrawn in EDTA tubes (BD Vacutainer™ Plastic Blood Collection Tubes) before surgical operation and maintained at room temperature and processed with Ficoll within a maximum of 1 h after collection. Isolated peripheral blood mononuclear cells (PBMCs) were stored at −80 °C until later examinations. Before storing at −80, PBMCs were counted using a hemocytometer and a concentration of 6000 cells per μl were prepared in order to minimize the cell count differences between samples.

RNA extraction, cDNA synthesis, and primer design

Of the prepared PBMC, 100 μl was directly used in RNA extraction process. After RNA extraction using TRIzol reagent and DNase treatment of extracted RNA, complementary DNA (cDNA) was synthesized using Revert Aid First Strand cDNA Synthesis Kit according to the instructions (Thermo Scientific, USA). Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Takara, Japan). Beta-actin was selected as reference gene and its primers were purchased from Qiagen Company (Hs_Actb_1_SG, QuantiTect Primer Assay QT00095431, Qiagen, USA). DCLK1 and Lgr5 primers were designed with AlleleID 6.0 software and were synthesized by Metabion Company (Germany). Their sequences are as follows:

DCLK1 F: AGGGTCGTAAACTGGTGGGAAAC
DCLK1 R: TGTCTGTATGGGCAAGATATGGTAAAC
and
Lgr5 F: CTGAACTAAGAACACTGACTCTGAATG
Lgr5 R: CACTTGGAGATTAGGTAACTGATTGC

Real-time PCR

Real-time PCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) for 1 cycle at 95 °C for 2 min followed by 40 cycles at 95 °C for 5 s

and 60 °C for 30 s. Specificity of products was verified by melting curve analysis (Fig. 1). Notably, each assay was done in triplicate and positive and negative controls were included in each run. Data was analyzed using comparative Ct method [50]. Also $2^{-\Delta Ct}$ where ΔCt is (CT gene of interest−CT internal control) was calculated in order to obtain mean±SD of each sample for further statistical analysis. Since the basic assumption of comparative Ct method is the approximately equal amplification efficiency of target and reference genes, average efficiency of each gene was calculated using real-time PCR Miner [51]. Conducting the experiment, comparable efficiency of β-actin (95.7), DCLK1 (94.8), and Lgr5 (96.6) approved the comparative Ct method for quantitative evaluation of DCLK1 and Lgr5 messenger RNA (mRNA) levels.

Study design and statistical analysis

Based on a designed prospective diagnostic case-control study, the main purpose of our study was to evaluate the mRNA expression level of Lgr5 and DCLK1 in isolated PBMC from CRC patients comparing to controls and further delineate the association of biomarkers expression level with patients' clinical and pathological features. In support of this, ΔΔCt was applied to get a fold change of gene expression level among patients and controls and parametric tests were used to assess the probable correlation between mRNA expression level of markers and clinicopathological characteristics. The correlation between Lgr5 and DCLK1 was measured by Pearson correlation coefficient test.

Based on our aims, the diagnostic performance of Lgr5 and DCLK1 as PB tumor markers was estimated by plotting receiver operating characteristic (ROC) curves and calculating area under the curve (AUC). Maximum point of Youden index was taken as an optimal cutoff of mRNA expression level separating case and control groups. Binary logistic regression was used to assess the diagnostic suitability of both markers together. IBM SPSS Statistics 22 was used for statistical analysis and a p value of <0.05 was considered statistically significant.

Results

The mean and standard deviation (SD) of Lgr5 and DCLK1 mRNA level in PB of CRC patients and controls was calculated using $2^{-\Delta Ct}$, as previously mentioned, and the related fold changes was obtained. In order to facilitate the calculation, decimals up to five digits have been removed. Based on our analysis, the mean of Lgr5 mRNA expression level was 363.2 (SD=115.1) in CRC patients and 75.3 (SD=33.6) in controls (Fig. 2). As a result, a 4.8-fold increase in the expression level of Lgr5 in blood of CRC patients was observed comparing to controls that was statistically significant ($p<0.001$). Additionally, Lgr5 expression level was significantly higher in patients at stages III and IV comparing to stages I and II ($p=0.031$). The significant elevation in Lgr5 expression level was also seen in high-grade tumors ($p=0.039$). CRT status had no significant effect on the expression of Lgr5 in PB of CRC patients. In addition, there was no significant correlation between the expression level of Lgr5 and other clinicopathologic findings (Table 2).

The mean of DCLK1 expression level in PB was 19.1 (SD=10.3) in CRC patients and 7.1 (SD=4.6) in controls (Fig. 2). Accordingly, DCLK1 mRNA level showed a 2.7-fold increase in PB of CRC patients compared to controls ($p<0.001$). DCLK1 mRNA level was significantly higher in stages III and IV compared to stages I and II ($p=0.025$). Moreover, a significant correlation was observed comparing the DCLK1 expression level in patients with and without the history of neoadjuvant therapy. Further analysis proved that DCLK1 was upregulated in patients with the history of neoadjuvant therapy ($p=0.041$). All statistical analysis is summarized in Table 2 based on calculated p value.

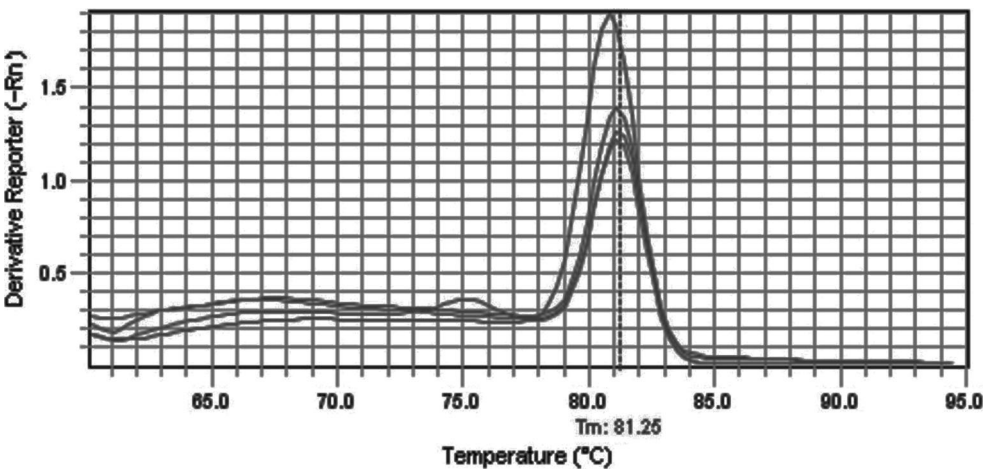

Fig. 1 DCLK1 melt curve analysis

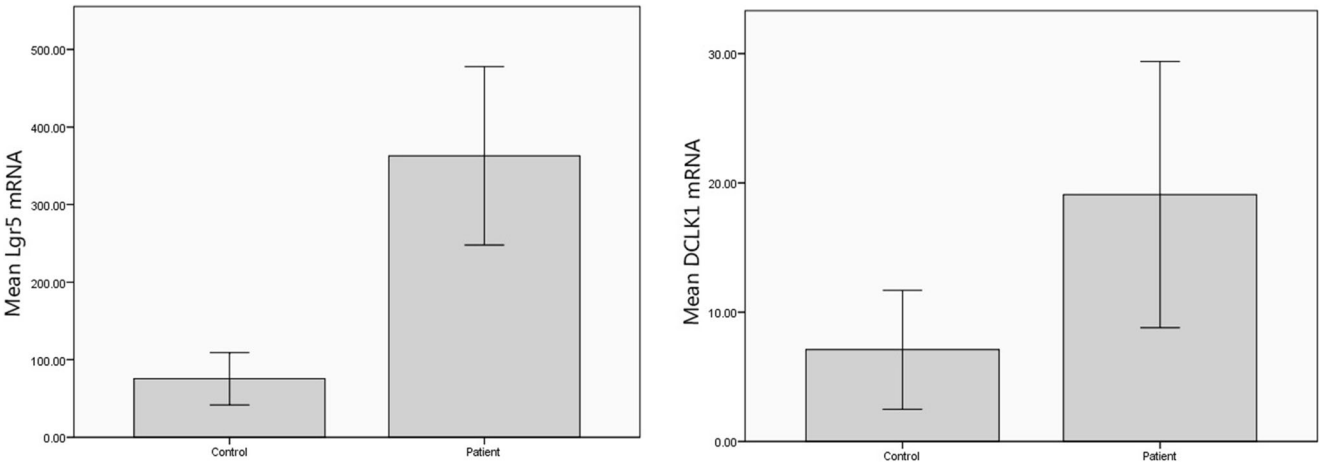

Fig. 2 qRT-PCR analysis of DCLK1 and Lgr5 mRNA expression level in CRC patients compared to healthy controls. Data are shown as mean±SD

Diagnostic test evaluation was considered based on sensitivity and specificity by constructing receiver operating characteristic (ROC) curve and further calculating area under the curve (AUC). The optimal cutoff for mRNA expression level that separate CRC patients from control was also determined. Comparing expression level of Lgr5 in patients and controls, the AUC was 0.865 (95 % CI, 0.783–0.925; p=0.001) (Fig. 3). In accordance to Lgr5 expression level, the optimal cutoff value (Youden index) was defined as 145.6 and the sensitivity and specificity of 74.2 and 90.5 were achieved, respectively.

Table 2 Correlation of clinicopathologic findings with Lgr5 and DCLK1 expression showed by p value

Parameter	Groups	Lgr5	DCLK1
Age (years)	20 to 40 40 to 60 ≥60	0.42	0.10
Gender	Female Male	0.32	0.36
Tumor location	Colon Rectum	0.23	0.12
TNM stage	I and II III and IV	0.031	0.025
Grade	Low High	0.039	0.06
Number of metastatic sites	0 1 ≥2	0.14	0.09
Lymphatic invasion	Unknown No Yes	0.21	0.08
Neoadjuvant history	Yes No	0.18	0.041

As for DCLK1, the ROC curve showed an AUC of 0.748 (95 % CI, 0652–0830; p=0.001) (Fig. 3). The DCLK1 mRNA level of 4.7 was defined as cutoff point and the sensitivity and specificity of 81 and 58, respectively, was determined in the mentioned cutoff value.

Combination of DCLK1 and Lgr5 markers was analyzed by logistic regression and predicted probabilities of diagnosis were used to generate a "combination marker" ROC curve. The combination (DCLK1/Lgr5) had an AUC=0.885 (95 % CI, 0.817–0.947; p=0.001) (Fig. 3). The sensitivity and specificity of the combination were 78 and 90, respectively. Consequently, combination marker may act more efficiently comparing to each single marker.

Correlation analysis was assessed using Pearson's correlation coefficient test in order to determine the association of Lgr5 and DCLK1 values. The results showed a weak positive correlation of 0.305 that was statistically significant (95 % CI, 0.1159–0.4734; p=0.002).

Discussion

Following the emergence of CSC hypothesis and its potential roles in tumor progression, metastasis, recurrence, and resistance to treatment, many efforts have been devoted to explore the implication of CSCs in prediction, prognosis, and treatment of various kinds of tumors including colorectal cancer [4]. A more recent interest has focused on circulating CSC (CCSC) markers based on the fact that PB is a suitable sample for investigating tumor markers as it benefits from noninvasiveness, simplicity of sampling, and affordability [52, 53]. Iinuma et al. investigated the mRNA expression level of CD133 as a proposed CSC marker in PB of CRC patients and reported it as a significant prognostic marker in patients with Dukes' B and C of colorectal cancer [54]. Fan et al. investigated prognostic significance of $CD45^{-}/CD90^{+}$

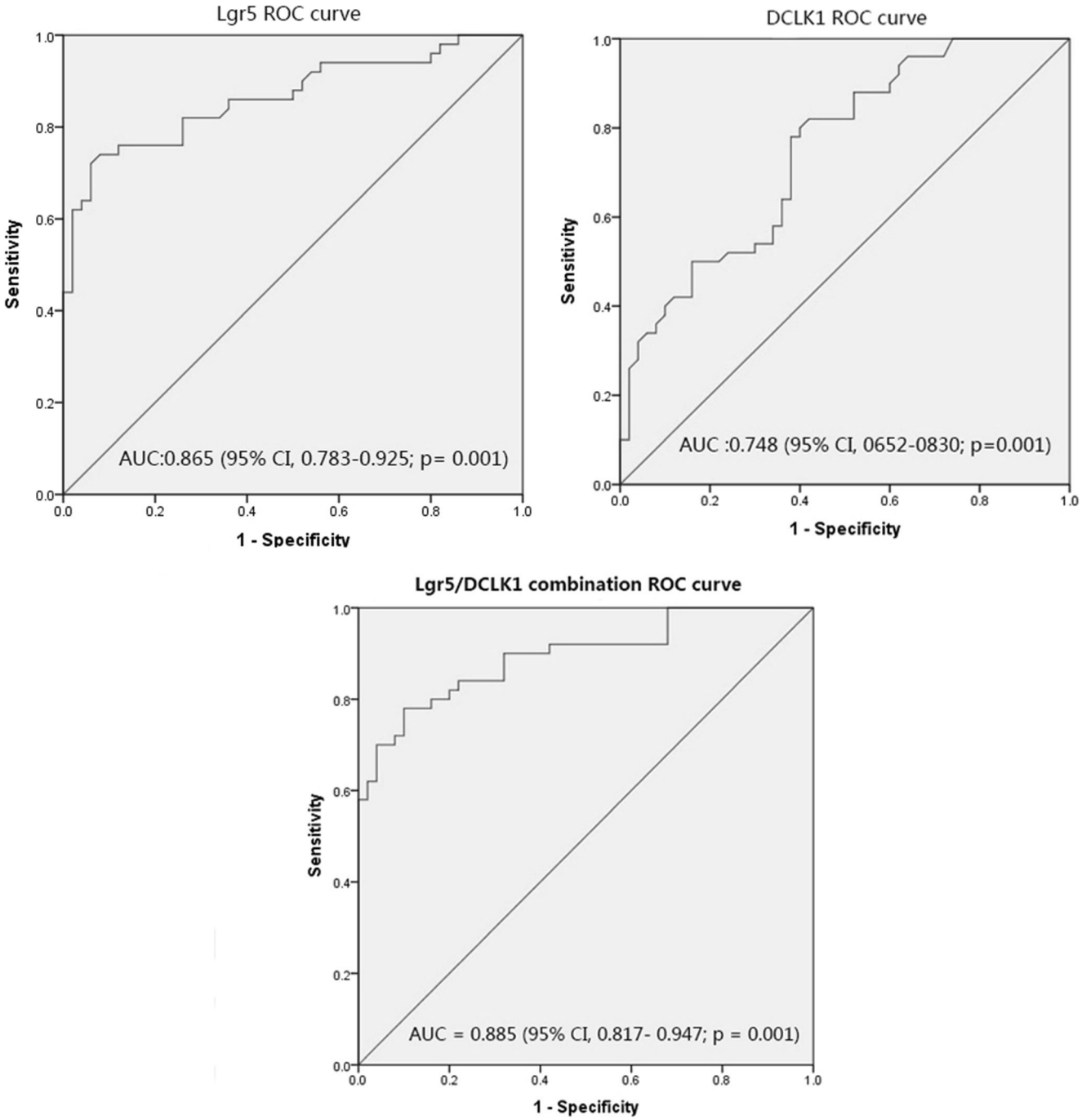

Fig. 3 Separate and combined Lgr5 and DCLK1 ROC curves. Area under the curve, 95 % confidence interval, and *p* values are shown

subpopulation as a potential CCSC marker in PB of patient suffering from hepatocellular carcinoma (HCC) and revealed that patients with CCSC ≤0.01 % showed significantly more favorable prognosis than those with CCSC >0.01 % [55]. Pilati et al. studied the prognostic value of putative circulating cancer stem cells in patients undergoing hepatic resection for colorectal liver metastasis and concluded that $CD133^+$ CTC may emerge as a suitable prognostic marker to stratify the risk of these patients [56]. The correlation between Lgr5 expression level and clinicopathological features as well as clinical outcome of CRC patients has been discussed in depth throughout the literature [37–39]. In addition, the diagnostic and prognostic significance of circulating Lgr5 as a potential marker of CRC in PB has been recently reported [57]. Moreover, accumulating evidences have emphasized on the role of DCLK1 as a candidate of CSC markers in CRC patients [44, 45]. To our knowledge, this is the first study investigating DCLK1 mRNA expression level in blood circulation of CRC patients. In addition, this would be the first study comparing the expression pattern of DCLK1 and Lgr5, as the most

remarkable CSC markers of CRC, in PB. Regarding DCLK1 as a marker of colorectal tumor stem cell and not normal intestinal stem cell, comparing the expression pattern of it to Lgr5 as a maker of both normal and cancerous intestinal stem cell marker [32] might give rise to valuable information that may possibly help future therapeutic and diagnostic implications of CSCs of colorectal cancer.

Our finding confirmed the previous studies indicating higher expression level of Lgr5 in PB of CRC patients compared to control group [57]. We also confirmed that Lgr5 expression was significantly higher in higher stages of CRC.

Based on previous histological analysis [43], overexpression of DCLK1 in PB of CRC patients was expected accordingly. As expected, our result showed a significant increase in DCLK1 mRNA level in PB of CRC patients compared to PB of controls. In addition, analyzing of samples based on the history of neoadjuvant therapy showed a significant increase in DCLK1 expression in patients with preoperative CRT history. Despite of many evidences indicating the relevance of Lgr5 expression pattern to tumor characteristics such as stage, prognosis, and survival [57, 58], the specific expression pattern of DCLK1 in neoadjuvant CRT-treated patients is perfectly suited to the concept of CSC. As previously discussed, dividing tumor cells are influenced by CRT and quiescent CSCs are resistant to such kind of therapeutic agents [17–20]. Regarding DCLK1 as a CSC marker that distinguishes CRC stem cell from normal stem cell, it could be concluded that CRT is unable to eliminate tumor cells containing DCLK1 marker. Consequently, in such patients, local and circulating tumor cells (CTCs), a combination of differentiated tumor cells, dividing tumor cells, and cancer stem cells would likely be enriched with CSCs. In other word, prior to CRT, tumor mass consists of a rare subgroup of CSC among a huge group of dividing tumor cells, whereas after CRT, the majority of dividing tumor cells has been eliminated by CRT, leading to CSC enrichment in both tumor tissue and blood circulation of CRC patients. Such phenomenon has been reported in several similar studies with different markers. Hamilton and Olszewski's study revealed the chemotherapy-induced enrichment of CSC in lung cancer [59]. Abubaker et al. showed an increased tumor burden as a result of single treatment of chemotherapy leading to the enrichment of ovarian cancer stem-like cells [60]. Dylla et al. reported colorectal cancer stem cell enrichment in xenogeneic tumors following chemotherapy [19]. Indeed, enrichment of CSCs in response to CRT is evidence that highlights CSC's clinical relevance. Likewise, the independency of Lgr5 expression pattern to CRT situation of patients may attenuate its proposed role as a potential CSC marker in CRC patients.

It is worth noting that since advanced tumors are more metastatic, more CSC will subsequently enter into blood circulation of patients with higher stages of the disease, and our study confirmed that the expression level of DCLK1 is elevated by increasing in the stages of CRC.

Although CSC enrichment following CRT may justify DCLK1 over-expression in CRT-treated patients, it should be noticed that over/under-expression of CSC marker is a multifactorial process. According to a great body of evidences, CSC fate is controlled by lots of complex functions such as epigenetic alterations including DNA methylation and histone modifications [61]. Several miRNAs cooperate with DNA methylation to regulate the balance between self-renewal and differentiation of CSCs [62]. Multiple observations indicate that maintenance of CSC features is orchestrated by these mechanisms leading to switching CSC markers on and off to generate heterogeneous CSCs with distinct phenotypes [61]. As a result, CSC phenotype may change in different phases of tumor in order to sustain tumor survival and growth and continuous alterations in CSC markers seems to be a programmed phenomenon. Consequently, although CRT did not show a significant effect on Lgr5 expression pattern, the probable role of Lgr5 as a potential CSC in CRC cannot be ignored and further investigations are still required to elucidate the different effects of CRT on DCLK1 and Lgr5 expression pattern.

The viewpoint that CSCs might switch their markers on and off will lead to a heterogeneous CSC population that makes the use of CSC markers more difficult. However, using a combination of CSC markers may solve the problem to some extent. Accepting this theory may explain why the DCLK1 and Lgr5 combination makes a slightly better marker, and it may even get better if we add some more CSC markers to this combination.

Other explanations for up/down-regulation of CSC markers are still remaining. For example, tumor cell dissemination and metastasis is dependent on epithelial-mesenchymal transition (EMT) process which converts adherent epithelial cells into migratory cells [63]. An increase in the proportion of immortalized human mammary epithelial cells exhibiting CSC markers was observed after EMT induction [64]. Moreover, emerging evidence suggests that resistance to chemotherapy is associated with acquiring CSC and EMT features [65]. In this regard, pancreatic cell lines that exhibited EMT features were resistant to chemotherapy agents and pancreatic cancer cells that were selected to be resistant to gemcitabine have exhibited EMT markers and increased invasion capabilities [66]. In other word, chemotherapy may affect the expression of some CSC markers such as DCLK1, while it may not affect other markers such as Lgr5. In addition, considering the effect of EMT on CSC, there might be a difference between local and circulating CSC markers and local CSC markers may not necessarily act as circulating CSC marker.

To further complicate the matter, Vedeld et al. surveyed a new aspect of DCLK1expression. Their study demonstrated that the DCLK1 promoter is hypermethylated in CRC patients

which results in down-regulation of DCLK1 in CRC patients, while no methylation has occurred in the normal mucosa samples [67]. However, chemotherapy situation of patients was not considered in their study; therefore, up-regulation of DCLK1 in patients who have undergone neoadjuvant therapy cannot be rejected by this data.

As previously mentioned, based on CSC concept, CSCs are the main tumor subpopulation responsible for CRT resistance. As such, a number of resistance strategies, including ATP-binding cassette (ABC) transporter expression, aldehyde dehydrogenase (ALDH) activity, B cell lymphoma-2 (BCL-2)-related chemoresistance, enhanced DNA damage response, and activation of signaling pathways, have been recognized for CSC drug resistance [17]. As a result, DCLK1 overexpression in chemoradiotherapy-treated CRC patients may be explained by CSC chemoradioresistance strategies leading to a new CSC clonal expansion resistant to CRT and methylation with higher DCLK1 expression.

Finally, even with all listed possibilities leading to different effects of CRT on Lgr5 and DCLK1, it could be simply related to the fact that DCLK1 only marks colorectal CSCs while Lgr5 is supposed to mark both colorectal normal and cancer stem cells, and the interaction between CRT and normal stem cells may affect Lgr5 expression pattern.

It is noteworthy that in both our study and Vedeld's study, the DCLK1 mRNA rather than DCLK1 protein has been investigated. Although mRNA and protein reasonably correlate to each other, results of many studies have proved that it is not always the case. As it is clear, mRNA will be destroyed after translation into protein, but proteins will remain in the cell until the end of its half-life. As a result, a CSC whose DCLK1 promoter has been hypermethylated and cannot express DCLK1 anymore may still carry DCLK1 proteins that have been built before hypermethylation. Several different studies confirmed that the mRNA level of a gene does not necessarily predict its protein level [68–71]. To this reason, the evaluation of DCLK1 protein instead of DCLK1 mRNA may result in more valuable information.

Moreover, the source of the markers in controls and patients should still be addressed in future studies. The question is if they are CSC markers, why do they appear in normal controls' circulation? There are many possible explanations to consider. It should not be neglected that although Lgr5 and DCLK1 are mentioned as the most colorectal CSC marker, it does not mean that they are not expressed by any other kind of cells. As an example, DCLK1 is mentioned as the brain-specific protein [72] and Lgr5 is frequently mentioned as gastrointestinal normal stem cell marker [36]. They might also be expressed by other tissues that have not been investigated yet, and they could enter the circulation for lots of unknown reasons such as in stem cell circulation process [73]. In addition, blood cells may themselves express these markers at basic levels that need to be elucidated in future investigations. On the other hand, a part of markers' expression in patients is related to other sources than CSCs. Fortunately, determining a cutoff gives us the capability to compare the expression pattern between controls and patients.

As a matter of fact, in order to determine the source of CSC markers in blood circulation, sorting of the cells based on multiple CSC markers is the most probable approach. In this way, we could consider $CD45^{-}/DCLK1^{+}/Lgr5^{+}$ cells as circulating colorectal CSC. However, CSCs constitute a small fraction of tumor cells, and from this amount [74], a very few number of CSCs enter the blood circulation in order to complete metastasis cascade and we only take 10 ml of blood as a representative sample. As a result, we need a high-throughput cell sorting method whose sensitivity is comparable to PCR.

Conclusion

Basically, we expect markers of the same category to move in the same direction. In this regard, we predicted a similar expression pattern for Lgr5 and DCLK1 as the proposed CSC markers. As expected, our data showed a significant level of similarity between Lgr5 and DCLK1 expression pattern in PB. However, some differences in expression pattern of markers considering patients clinicopathologic findings was observed. The most prominent difference was found in patients with and without the history of neoadjuvant therapy that increase in mRNA expression of DCLK1 but not Lgr5 was observed in patients with pre-operation chemoradiotherapy. This difference could be simply attributed to the nature of Lgr5 as it is both normal and CSC marker, whereas DCLK1 is considered as an exclusive colorectal CSC marker. Other factors may also associate to the observed differences in the expression of CSC markers that has been discussed in detail. Our results also showed positive diagnostic and prognostic role of Lgr5 and DCLK1 as potential CSC markers in CRC. However, application of these markers in clinical implications still needs more evaluation in a more comprehensive study with bigger sample number.

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